A Case-Control Study of Antibodies to *Toxoplasma gondii* and Risk of Schizophrenia among U.S. Military Personnel

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The views expressed are those of the authors and should not be construed to represent the positions of the Department of the Army or Department of Defense

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ABSTRACT:

Objective: A number of studies have reported associations between *T. gondii* infection and risk of schizophrenia. Most existing studies used small populations and single post-diagnosis specimens. As part of a larger research program we conducted a hypothesis-generating, pilot case-control study of *T. gondii* antibodies among individuals discharged from the US military with a diagnosis of schizophrenia and multiple pre- and post-diagnosis serum specimens available.

Methods: Cases (n=200) were military members discharged for a diagnosis of schizophrenia. Controls, 3:1 matched on several factors, were members not discharged. The military routinely collects and stores members' serum specimens. We used microplate enzyme immunoassay to measure IgG antibody levels in pre-diagnosis specimens.

Results: A positive association between IgG antibody and schizophrenia was found overall (HR = 1.38, 95% CI 1.17 to 1.64) as well as for hospitalized and non-hospitalized males and hospitalized females. The association between IgG and schizophrenia varied by the time interval between the specimen collection date and onset of illness.

Discussion: We found significant associations between increased levels of scaled *T. gondii* IgG antibodies and schizophrenia, for antibodies measured both prior to and after diagnosis. These findings will be used to design and execute a larger, hypothesis testing study among military personnel, with the potential for developing new methods of preventing and treating some cases of schizophrenia.

Key words: toxoplasma, antibodies, psychosis, schizophrenia, case-control, hazard, military, biomarkers

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Introduction

Schizophrenia is a chronic, debilitating neuropsychiatric condition with a prevalence of approximately 1.1% among adults in the U.S. (1). Most frequently diagnosed in young adults, it often causes severe disruption of education and career trajectories. Advances in psychopharmacology over the past few decades led to several treatment options, but their effectiveness is variable and limited to treating symptoms rather than baseline disease. While scientific research into the causes and manifestations of psychiatric disorders marked by both psychotic and mood symptoms has been ongoing for more than a century, the etiology of this illness remains uncertain (2).

Certain risk factors, however, have been established. These include both genetic and environmental factors such as family history/genetic predisposition, winter or spring birth month, urban birth, lower socio-economic status and prenatal or birth complication (2) (3). An infectious agent as the possible link between these various risk factors has been proposed for more than a century; a hypothesis supported by a growing body of recent scientific research (2).

Much recent research has focused on the potential relationship between infections with *Toxoplasma gondii*, a neurotropic parasite, and development of schizophrenia (4-6) Serum antibodies to *Toxoplasma gondii* have been found to be increased in cases as compared to controls in a number of different study populations (7-9). The possible link between Toxoplasma and schizophrenia is supported by the occurrence of psychiatric symptoms in some individuals with acute Toxoplasma infections(10) as well as by animal models documenting neurological and behavioral changes during acute toxoplasma infection (11, 12) .

Most of the studies published to date, however, have been limited by small sample sizes and single-specimen serologic data obtained after diagnosis. Studies of pre-onset serologic and demographic data on large numbers of effected individuals and appropriately matched controls are difficult to achieve because population-based medical and serologic data are not routinely collected and stored on adults. The U.S. military does, however, collect such data and samples from all active duty personnel. Serum samples are obtained at the point of accession and approximately every two years thereafter while they remain on active duty (13). Outpatient and inpatient medical encounter data is electronically archived and managed for both surveillance and research purposes (http://amsa.army.mil). This affords a unique opportunity to conduct large, population-based serological analyses on specimens obtained prior to diagnosis of disease. We report on the association between increased levels of antibodies to *Toxoplasma gondii* and subsequent risk of schizophrenia among active duty military personnel in a hypothesis generating pilot study.

Method

Subject and specimen selection: This project was reviewed and approved by the appropriate human protection committees at the authors' institutions. The methods of study subject selection and inclusion, serum selection and shipping, and sources and types of ancillary data are presented in Appendix A. Cases were defined as military members medically discharged from military service for a diagnosis of schizophrenia. All references to schizophrenia in Results and Discussion refer to service members discharged from the military with a diagnosis of schizophrenia. Date of illness onset was estimated by the first military hospitalization with a mental health diagnosis (ICD-9-CM codes 290-319). For subjects without such a hospitalization, date of onset was estimated to be 12 months prior to the final Physical Disability Agency disposition date to approximate the time from onset to disposition allowing for clinical evaluation and administrative processing. Controls were selected from the population of service members with no inpatient or outpatient diagnosis of a mental health disorder (ICD-9 codes 290-319). Controls were matched in a 3:1 ratio with cases on the following variables: date of birth (+/- one year), the corresponding case's accession date +/- six months, sex, race (Black, White, Other), and branch of military service.
Laboratory: IgG antibody to *T. gondii* was measured by microplate enzyme immunoassay as previously described (8). Enzyme immunoassay consists of the binding of serum to solid phase *Toxoplasma* antigen, derived from the RH strain of *Toxoplasma gondii*, and subsequent reactions with enzyme labeled anti-human IgG and enzyme substrate. The amount of color generated by the enzyme substrate reaction was measured in optical density (OD) units by means of a microplate colorimeter. This method of analysis was selected because it allows for high throughput measurement of antibodies using a common platform and requiring only small amounts of sample. *T. gondii* reagents were obtained from Viro-Immun Labor-Diagnostika GmbH Oberursel, Germany. Standard samples consisting of known amounts of Toxoplasma antibodies expressed in international units were run on each microwell plate. Samples were run under code in matched groups in which case and control status were not identified and were not known by the laboratory performing the assay.

Quantitative Antibody Measurements Data Normalization:

All matched case-control samples were tested on the same plates; over the course of the study samples were assayed on 33 different plates. To control for potential systematic error introduced by plate-to-plate variation, data were normalized using the robust median normalization method which combines the within plate and between plates variance, as shown in equation 1,

$$(1) \qquad S_{ijk} = \frac{R_{ijk} - M_k}{\sqrt{V_k + V_b}}$$

where R_{ijk} is the raw optical density of a subject's blood sample and S_{ijk} is the scaled score for that sample which was in plate k, the ith case-control group and the jth blood

sample. M_k is the median of all $\{R_{ijk}\}$ of the control samples in the plate k, V_k is the variance of R_{ijk} of control samples in the plate k, and V_b is the variance of all R_{ijk} between plates. When the raw score is homogeneous across the plates, the variance between plates is zero. After the data was normalized, the index of each plate could be omitted. We use S_{ij} to represent the blood sample from the ith subject and jth sample in the subsequent discussion.

The conditional logistic model was used to verify linearity of the association between schizophrenia and the antibody level. The model is shown in equation 2:

(2) If_case_{ij} = age + race + gender+ mental health hospitalization + Hispanic +branch of service + years service+ years from specimen collection to diagnosis + S_{ij} + interaction terms for antibody level as a continuous variable.

When the main effect of the antibody level showed strong effects, we modeled the following interaction terms: demographic factor* S_{ij}, the time to diagnosis* S_{ij} and the category of S_{ij} * S_{ij}. The interaction estimates the heterogeneity of the agent effect, which may vary by demographic variable or by time, as well as the non-linearity of the agent effect. Whether the interaction should be included in the final model depended on its significance. Since the antibody levels for all specimens for each subject were included, the inter-dependency of longitudinal data among individuals was controlled.

Results

A total of 200 cases and 591 controls were included in the study population. Nine cases could only be matched to two controls. Table 1 shows the distribution of case and controls by those demographic factors. Overall, about 83% were males, about 50% were younger than 25, 12% were older than 35, about 10% were Hispanic, 90% had a mental health hospitalization, and over 50% were in the Army. Approximately 75% of cases had less than 5 years of military service and 90% had at least one mental health hospitalization.

Conditional logistic models were run to evaluate the effect of scaled antibody levels on the schizophrenia hazard ratio, as well as to control for the matched and un-matched factors and the inter-dependency of blood samples within the same subject and matching groups. The overall hazard ratio for IgG was 1.38, (95% CI 1.17 to 1.64).

In order to check the homogeneity of the scaled IgG effect by gender and mental health hospitalization, the two-way interactions of gender and IgG, mental health hospitalization and IgG, and the three-way the interaction of gender, mental health hospitalization and scaled IgG were included the model. The three-way interaction is mildly significant with a p value of 0.09 which suggest that the heterogeneity of the scaled IgG effect across the gender and hospitalization status should not be ignored. (Data is not shown pending review for publication).

The hazard ratios for men, both those with and without mental health hospitalization, and for women with mental health hospitalization were essentially the same. For hospitalized subjects only we evaluated the effects of when the specimens were collected in relationship to the estimated date of onset (Data is not shown pending review for publication). Six different time periods between specimen collections to diagnosis were defined: more than three years, between two and three years, between one and two years, six months to one year, within six months of diagnosis, and after onset of illness. The controls' specimens corresponded to the same time periods, relative to the matched case's diagnosis date. Different effects were noted for different periods (Data is not shown pending review for publication). The HR was significantly greater than 1.0 for those specimens collected greater than three years before diagnosis, and for those collected within six months prior to or after diagnosis.

Discussion

This hypothesis-generating pilot study found consistent associations between IgG and risk of schizophrenia for both men and women with a mental health hospitalization. The magnitude of the hazard ratio was similar for all men and hospitalized women. The HR also varied by the duration of time between the specimen draw and the time of onset of illness. Since our study population had serum stored in a repository, we were able to retrieve and analyze samples that were obtained from apparently healthy individuals prior to disease diagnosis. This study design allowed us to document that elevated levels of Toxoplasma antibody existed prior to symptom onset, making it very unlikely that the observed antibody level elevations were simply an artifact of disease-related exposures or a genetic predisposition to disease. Furthermore, our study design allowed for the close matching of cases and controls on demographic variables such as race, ethnicity, and age, making it unlikely that the case-control differences were related to population stratification or other artifactual differences in the two populations.

There are several limitations to the current study. Due to the sample size, the data are inadequate to evaluate the impact of changes in scaled IgG levels over time for most individuals in the study population. Furthermore, because the proportional hazard (PH) model weights each serum specimen equally it may be calculating an increased HR for subjects who had both temporally distal as well as proximal elevated scaled IgG levels. There was also substantial variability of scaled antibody level over time within individuals. Appropriate interpretation of the findings is not obvious and may be at least partially model-driven.

Military populations with schizophrenia differ somewhat from other populations in being older at age of onset of schizophrenia, most likely due to self-selection and to medical evaluation and screening prior to entry. This selection process results in few persons with overt psychosis entering the military, and therefore these findings may limit the generalizabilty to the broader population of Americans with schizophrenia. Although our study relied on data obtained through electronic medical records, each case selected for the study was subjected to a thorough medical and clinical review by at least one psychiatrist and two other physicians, usually including another psychiatrist, during the military medical discharge process. In addition, preliminary findings from an independent record review of study subjects' hard copy medical records demonstrate a greater than 90% verification of diagnoses (manuscript in preparation) indicating that diagnostic misclassification is not likely to be a major source of bias. Given the pre-induction medical screening process, it is unlikely that there are many or any false negatives among our control population, and any false positive errors that did occur would most likely be independent of scaled IgG level and therefore would bias our findings towards the null.

Our results are consistent with previous studies indicating an increased level of antibodies to *Toxoplasma gondii* in individuals with recent onset schizophrenia (7, 8). However, in previous studies relying on the identification of new cases of schizophrenia, *Toxoplasma* antibodies were not measured until after diagnosis, raising the possibility that the increased levels of antibodies were the result of hospitalization, medications, or other

disease-related environmental factors(14). Our study offers a clear improvement on this aspect.

Given the high prevalence (over 20%) in the United States (15), it is clear that most individuals infected with *T. gondii* do not have schizophrenia. The reasons for differential reactions to *Toxoplasma* infection is not known with certainty but may be related to genetic determinants of host susceptibility (16, 17) or to varying degrees of pathogenicity among infecting organisms (18). Furthermore, there may be differential responses to infection acquired from contact with cat feces, which contain sporozoites in the form of oocysts, as compared to infection resulting from the ingestion of *Toxoplasma* infected meat, which contain bradyzoites residing in tissue cysts (19). The timing of infection may also be an important differential risk factor. Previous studies have indicated that children born to mothers with increased levels of antibodies to *Toxoplasma gondii* are at risk for the development of schizophrenia in later life (20). Our study design did not permit us to distinguish perinatally acquired primary *Toxoplasma* infection from infection acquired in later life. Additional studies of cohorts, including ones followed from birth should be employed to define the timing of *Toxoplasma* infection and the risk subsequent risk of developing schizophrenia.

The mechanisms by which exposure to *Toxoplasma gondii* might lead to schizophrenia are also not known with certainty. However it is of note that animals experimentally infected with *Toxoplasma gondii* show altered behavior including abnormal reaction to novel environmental stimuli (21). Altered novelty seeking behavior has also been found in humans with Toxoplasma antibodies (22, 23). Additional studies should also be performed in humans and animals to document pathophysiological mechanisms associated with *Toxoplasma* infection and altered behavior.

Recent studies have documented the limitation of currently available medications for the treatment of schizophrenia and have highlighted the importance of finding new methods for early diagnosis and treatment (24, 25). *Toxoplasma* infections in the United States are largely preventable by public health measures directed at the avoidance of organisms shed by infected cats and by the eradication of infectious food borne cysts by proper cooking and handling (12), (23). There are also a number of medications with in vivo and in vitro anti-*Toxoplasma* activity, including some medications used to treat individuals with schizophrenia (26). The efficacy of such interventions should be investigated by appropriately designed clinical trials; such trials might lead to novel methods for the prevention and treatment of schizophrenia and other devastating human neuropsychiatric diseases.

This pilot study is part of the largest and first longitudinal study in the literature to date of the association of selected infectious disease and in particular toxoplasmosis with schizophrenia. The findings in this paper will be confirmed in a future hypothesis testing study with approximately 400 identified cases of schizophrenia and over 1200 longitudinal pre- and post-diagnosis serum specimens. The future study will also investigate genetic-environmental interactions as they relate the etiology of schizophrenia.

References (available upon request)

Table 1. Description of Military New Onset Psychosis Project (MNOPP) Study Subjects

Characteristic	Cases n (%)	Controls n (%)
Sex		
Male	165 (82.5)	490 (82.9)
Female	35 (17.5)	101 (17.1)
Age Categories		
≤20	32 (16.0)	101 (17.1)
21 - 24	69 (34.5)	204 (34.5)
25 - 29	48 (24.0)	139 (23.5)
30 - 34	27 (13.5)	75 (12.7)
≥35	24 (12.0)	72 (12.2)
Race		
White	99 (49.5)	294 (49.8)
Black	89 (44.5)	263 (44.5)
Other	12 (6.0)	34 (5.8)
Hispanic		
Yes	21 (10.5)	37 (6.3)
No	179 (89.5)	554 (93.7)
Branch of Military		
Army	106 (53.0)	312 (52.8)
Air Force	22 (11.0)	66 (11.2)
Marines	15 (7.5)	45 (7.6)
Navy	57 (28.5)	168 (28.4)
Time in Service Categories (years)		
≤ 1	56 (28.0)	139 (23.5)
> 1 to < 3	61 (30.5)	180 (30.5)
≥ 3 to < 5	33 (16.5)	122 (20.6)
≥ 5 to < 10	29 (14.5)	87 (14.7)
≥10	21 (10.5)	63 (10.7)
Mental Health Hospitalization[a]		
Yes	180 (90.0)	532 (90.0)
No	20 (10.0)	59 (10.0)

[a] Cases with and without mental health hospitalization and matching controls